

Biochar and manure amendments impact soil nutrient contents and microbial enzymatic activity in a semi-arid irrigated maize cropping system

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## **Abstract**

To mitigate negative impacts on crop yield from water-saving limited irrigation, we tested the effect of two organic amendments on soil water holding capacity and nutrients. In an experimental maize field in northern Colorado, we tilled in conventional steer manure (30 Mg ha<sup>-1</sup>) and a fast-pyrolysis pine-wood biochar (30 Mg ha<sup>-1</sup>). We quantified impacts on soil moisture, total carbon and nitrogen, mineral nitrogen, available phosphorus, microbial biomass, and seven extracellular enzymatic activities (EEAs). Compared to the control, manure amendments increased gravimetric soil moisture by approximately 15%, total nitrogen by 10%, available phosphorus by 45%, and microbial biomass carbon by 15% ( $p < 0.05$ ). Relative to the control, biochar increased total soil carbon by 80% and altered EEAs ( $p < 0.05$ ). Biochar significantly increased  $\alpha$ -1,4- glucosidase,  $\beta$ -D-cellobiohydrolase and  $\beta$ -1,4-N-acetylglucosaminidase and significantly decreased  $\beta$ -1,4-glucosidase and phosphatase activities ( $p < 0.05$ ). Despite the effects on soil moisture, nutrient availability, and microbial dynamics, neither amendment significantly impacted maize yield under limited irrigation. Ongoing measurements will allow us to fully assess longer-term impacts on yield.

Key Words: biochar; manure; extracellular enzymes; irrigation; microbial nutrient cycling; corn

## **1. Introduction**

Increasing drought and competition for water resources among municipal, industrial, and agricultural sectors requires improved water conservation in semi-arid regions. Agricultural producers can reduce water use by adopting limited irrigation strategies (DeJonge et al., 2011; Fereres and Soriano, 2007), such as applying irrigation only at critical crop growth phases (Schneekloth et al., 2009). To enhance soil water retention under limited irrigation, traditionally, producers have treated soils with organic amendments, such as manure (Bulluck III et al., 2002). Alternatively, recent research indicates that a charcoal-like amendment, known as biochar, can have similar effects under limited irrigation, increasing soil volumetric moisture content even with reduced water inputs (Akhtar et al., 2014).

Biochar is created through the pyrolytic conversion of any organic feedstock in an oxygen limited environment, at temperatures  $>250^{\circ}\text{C}$  (Lehmann and Joseph, 2015). The resulting product consists of highly stable condensed aromatic carbon (C) rings, with physiochemical characteristics that depend on pyrolysis conditions and feedstock type (Enders et al., 2012). This high variability in characteristics necessitates the investigation of different biochars in various soil types and climatic regions to determine which biochar can effectively achieve particular management goals (Novak et al., 2014). For example, use of biochar co-generated from bioenergy production from local feedstock in our study system in Colorado may also have additional environmental and cost benefits (Field et al., 2013) compared to conventional amendments.

With high organic C content, both manure and biochar could have similar impacts on soil nutrients, structure, and microbial dynamics in agricultural systems. Soil fertility research has long established that manure amendments add nutrients to soils (*e.g.* organic N or ammonium ( $\text{NH}_4^+$ )) and also improve soil structure, therefore increasing nutrient retention and water holding capacity (Salter and Williams, 1968;

Ware and Johnson, 1949). These changes can benefit crop production by improving nutrient cycling via stimulation of microbial growth and activity (Bulluck III et al., 2002; Elzobair et al., 2016; Peacock et al., 2001). Active soil microbes metabolize and turnover organic matter, secreting specific extracellular enzymes to break down large organic molecules into monomers, also available for plant uptake (Burns, 1982). Since specific enzymes are known to cycle C, N, and P substrates, shifts in extracellular enzymatic activities (EEAs) are often used as a proxy for changing metabolic pathways and thus microbial function in soils (Bell et al., 2013). The addition of organic material such as manure increases available C in the soil, which causes growth in microbial biomass (Witter et al., 1993), and can increase the production and activity of extracellular enzymes (Burns et al., 2013a). As a high C substance, with a large surface area and porosity, biochar also has the potential to similarly influence soil structure and nutrient retention, and thus microbial biomass and subsequent enzymatic activity.

In temperate agriculture, biochar addition influences microbial dynamics through physical changes to soil structure and through chemical changes to soil stoichiometry and pH (Ippolito et al., 2012; Lehmann and Joseph, 2015; Quilliam et al., 2013; X. Wang et al., 2015). By augmenting soil surface area and porosity, biochar can increase soil water holding capacity (Brockhoff et al., 2010), and provide habitat and relief from predation for microbes (Jaafar et al., 2015). Aside from physical habitat, biochar's large surface area and reactivity attracts ions and low-molecular weight organic compounds; thus biochar can initially increase nutrient retention and potential sites for microbe-substrate interactions (Gul et al., 2015). Even with these known structural changes, the effect of pine-wood biochar on microbial biomass remains variable, ranging from no impact to 100% increases (Brantley et al., 2015; Domene et al., 2014; Gomez et al., 2014; Jin, 2010). Despite the wide variation in response of microbial biomass to biochar, few researchers have quantified how microbial function is altered by these induced soil physical changes in soil moisture and surface area, and chemical changes to nutrient retention and pH (Elzobair et al.,

2016; Lehmann et al., 2011; Oleszczuk et al., 2014). Previous methods have quantified microbial functional shifts and changes to nutrient cycling by measuring EEAs (Bell et al., 2013; Burns et al., 2013b).

Enzyme activity is sensitive to pH and typically changes with nutrient dynamics, so quantification of these proteins can further our understanding of biochar's impact on microbial function and overall soil fertility. Current research on biochar-enzyme interactions assesses soil chemistry and stoichiometry. Since biochar can influence soil pH (alkaline pine biochar can lime soils by 1.0-1.4 units (Rogovska et al., 2014)), it can impact enzymatic activities that function within restricted pH ranges. The pH effect depends on the chemical composition of the biochar, which also can influence soil nutrients. Biochar addition alters soil stoichiometry due to the large organic C inputs. This increase of C in some temperate ecosystems can lead to an increase in microbial nitrogen (N) immobilization into biomass by up to three-fold (Güereña et al., 2012) and subsequent N stabilization on biochar surfaces (Brantley et al., 2015), although biochar addition does not always induce N immobilization (M.L. Cayuela et al., 2013). Biochar effects on N dynamics and N-cycling enzymes remains ambiguous (Bailey et al., 2002), as even soil N mineralization has been shown to decrease (Lentz et al., 2014), increase (Domene et al., 2014), and remain unchanged (Gaskin et al., 2010) after biochar addition. Recent biochar studies also show variable impacts on soil P: one greenhouse trial demonstrated no impact on soil P (Domene et. al 2014), a short incubation suggested biochar alters colloidal particles and P retention (Soinne et al., 2014), and another column study suggested that biochar lowered P bioavailability due to adsorption of orthophosphate and organic P compounds to its surface (Laird et al., 2010). Yet another consideration for nutrient stoichiometry and enzyme interactions is that biochar contains a small labile component that can provide readily available nutrients for soil microbes and stimulate activity (Anderson et al., 2011; Lehmann et al., 2011; Spokas et al., 2012; Warnock et al., 2007). These variable nutrient dynamics from

laboratory and column studies prompt the examination of biochar and microbial nutrient dynamics within specific systems *in situ*.

We compared the effects of manure and bioenergy co-generated, fast-pyrolysis pine biochar amendments on maize yield under limited irrigation. We specifically analyzed soil moisture, microbial abundance, and enzymatic activities to assess changes to soil structure and microbial function. We hypothesized that the biochar would increase soil moisture and total soil C. Due to these increases, we predicted that biochar would enhance microbial biomass, increasing enzymatic nutrient cycling, and thus more effectively maintain yield even under limited irrigation. We predicted manure to have similar, but weaker effects in this semi-arid temperate agricultural system.

## **2. Methods**

### **2.1 Field site and experimental design**

This experiment was conducted at the Agricultural Research Development and Education Center, Colorado State University, Fort Collins (40.59°N, 105.14°W, 1560 m elevation). The climate is semi-arid, with an annual rainfall of 408 mm (average normal from 1981-2010) (usclimatedata.com, accessed 2016). After biochar application in October of 2013, the rainfall during the maize growing season (May 1-Oct 31, 2014) was 272 mm, and average air temperature 16.3°C, ranging between -3.44°C and 35.8°C (Colorado State University CoAgMet weather station, 40.65°N, 105°W). The soil is classified as a Fort Collins loam, with 51% sand, 20% silt and 29% clay (Abulobaida, 2014). The soil was further characterized by Abulobaida (2014) as having a 1.3g cm<sup>-3</sup> bulk density, CEC of 24.65 nmol<sub>c</sub> kg<sup>-1</sup>, 1.5% total C, 0.1% total N, 185.0 ppm K, 514.75 ppm Mg, 3904.458 ppm Ca, 81.458 ppm S, 0.583 ppm Zn, 2.242 ppm Mn, and 575.5 ppm Fe.

We implemented a split-split plot design with four replicate blocks. The main plots were “full” and “limited” irrigation treatments, further split into two maize hybrid subplots. We then applied three soil organic amendment treatments: biochar, manure, and a control, with no amendment, for a total of 48 sub-subplots. Soil samples were analyzed at the sub-subplot level (n=4). Each amended plot was 4.5m x 4.5m and planted with six rows of maize. A nine m alley separated the main irrigation plots on all sides.

The field was prepared in September of 2013 by tilling to 30cm, followed by leveling in October. The pine-wood biochar and steer manure (Table 1), were surfaced applied at 30 Mg ha<sup>-1</sup> (dry weight) and were disc-tilled in to 15cm on November 14, 2013. The biochar consisted of primarily virgin pine wood, that underwent fast-pyrolysis for energy generation at 400-700°C with five minutes of reaction time (Confluence Energy LLC, Kremmling, CO). Biochar and manure properties are reported in Table 1, including: total C and N, pH, bulk density, cation exchange capacity (CEC) (measured using an ammonium acetate protocol), Brunauer-Emmett-Teller surface area and pore volume (measured with N<sub>2</sub> gas) (see other laboratory methods below). The pine biochar had a particle size of 0.25-3.0mm and a moisture content of 49.2% at the time of application. Further analysis by Control Laboratories characterized the biochar as 10.8% ash, 4.5% O, 1.3% H, 0.38 % total P, 0.06 O:C ratio, and 0.21 H:C ratio (Watsonville, CA).

Irrigation occurred once per week for the well-watered “full” irrigation determined by the difference between accumulated evapotranspiration and precipitation during the week. For the “limited” irrigation treatment, all irrigation inputs were withheld from the appearance of the seventh collared leaf (V7) to maize tasseling (*i.e.* June 29 – July 28, 2014). This resulted in a 30% reduction in irrigation inputs.

Fertilizer was applied at 202N-45P-13S-1Z kg ha<sup>-1</sup> on April 9, 2014, and tilled with a roller harrow to 10 cm. Herbicide and side-dressing were applied on June 17, 2014 at the following rates: Roundup® PowerMAX at 2.3L ha<sup>-1</sup>, status herbicide at 0.33L ha<sup>-1</sup>, Ad-Wet 90 at 0.35 L ha<sup>-1</sup>, and Ammonium Sulfate sprayable at 0.49 kg ha<sup>-1</sup>. On May 19, 2014 two Dupont® Pioneer maize hybrids (P8954AM and P9305AM) were seeded at approximately 79,000 seeds ha<sup>-1</sup> with row spacing of 76 cm. Except for percent cob fill (see results), we did not observe significant differences between the two hybrids and results are averaged across them (n=8).

## 2.2 Soil sampling and characterization of soil moisture and nutrients

We sampled soils from 0-10 cm with a 2.5 cm-diameter hand corer on the following three dates in 2014: (A) June 29, 41 days after planting (before the start of the limited irrigation drought period), (B) July 27, 69 days after planting (at the end of the limited irrigation treatment), and (C) September 1, 105 days after planting (three weeks before the final harvest). Four random cores were taken to create one bulked sample per sub-subplot. Soils were sieved to 2 mm and stored in plastic bags and kept at ~4°C until final analyses, which occurred within a few days from sampling.

Lab analyses to characterize basic properties on all samples included gravimetric soil moisture, total soil C and total soil N content, pH, available P, and mineral N (ammonium (NH<sub>4</sub><sup>+</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>)).

Gravimetric soil moisture was determined on a 10 g subsample by drying in a 105°C oven for 24 h, and subsequently used for soil dry weight correction. The oven-dry soil samples were pulverized and used for the analyses of %C and %N on a LECO True-Spec CN analyzer (Leco Corp., St. Joseph, MI, USA) and for pH measurements in 1:5 deionized water using an Orion EA 9110 Meter (Thermo Scientific, Beverly, MA, USA). Available P was measured with the molybdenate blue method for alkaline soils (Dick and Tabatabai, 1977). Mineral N was extracted from field moist 15 g soil subsamples with 75 mL of 2M KCl



for analysis on an Alpkem Flow Solution IV Automated wet chemistry system (O.I. Analytical, College Station TX, USA) (Miller and Keeney 1982). All analyses were completed at the EcoCore Analytical Facilities (Colorado State University, Fort Collins, CO). Additionally, using a subset of samples from November 2013 (n=3 per soil treatment), we measured plant available water with a pressure plate apparatus (Klute, 1986) as the difference between field capacity (-33 KPa) and wilting point (-1500KPa) (data not shown).

### 2.3 Microbial biomass and enzymatic activities

All soil samples were analyzed for microbial biomass and EEA within 10 days from collection. Microbial biomass was determined using two 8 g subsamples via the chloroform fumigation extraction method, modified to a 5 day incubation period (Brookes et al., 1985), with extractable total organic C and total N measured with a TOC-V-TN analyzer (Shimadzu Corp., Kyoto, Japan). As a proxy for microbial biomass, we used the chloroform fumigation-extracted C and N, referred to as microbial biomass C (MBC) and microbial biomass N (MBN). We tested for sorption of dissolved organic C and N onto the biochar surface (Jin, 2010) by adding biochar at the same rate of field application to five control soil samples directly before the fumigation. The calculated correction factors for sorption of dissolved organic C and N on the surface of the biochar had no significant impact on MBC and MBN results; thus uncorrected values are reported.

To assess potential EEA we used a high-throughput fluorometric assay for seven common extracellular enzymes (Table 2) that are known to breakdown C, N, and P substrates, as described by Bell et al. (2013). Briefly, 91 mL of 50 mM Tris buffer (pH 8.3) was blended with 2.75 g of field moist soil for one minute. From this soil slurry, 800 µL was filtered and pipetted into a 96 deep-well plate, with seven rows filled with 200 µL of 200 µM substrate (Table 2). For each sample two standard plates were made with

soil slurries (800  $\mu$ l) mixed with the two fluorogenic moieties without substrate, at concentrations from 0 to 100  $\mu$ M (Table 2, 4-Methylumbelliferone and 7-Amino-4methylcoumarine). From these two standard plates, we calculated curves to correct for quenching of fluorescence due to floating soil or organic particles. Each plate of soil-substrate mixture and corresponding two standard plates were incubated for 3h at 22°C. After incubation, the plates were centrifuged at 1500 RPM for 3 minutes and 200  $\mu$ l of supernatant was transferred to a new 96-well black plate. The absorbance was measured at 365 nm excitation and 450 nm emission on an Infinite M200 Microplate Reader (Tecan Trading AG, Switzerland).

#### 2.4 Maize biomass and yield

Plant aboveground biomass was measured on September 22, 2014 after the crop reached physiological maturity. All plants in a 2 m section of each sub-subplot were cut at the ground and biomass was weighed moist in the field. We then hand-harvested one plant per plot to calculate the moisture correction and grain biomass, by drying at 70°C and weighing each plant (Hay, 1995). Yield data are reported in Mg ha<sup>-1</sup> dry grain and dry biomass (including grain, cob, stalks, and dropped leaves). Percent cob fill was measured as the length of the cob with kernels divided by the whole cob length. The harvest index was calculated as the grain mass over the total plant biomass.

#### 2.5 Statistical Analysis

All statistical analyses were conducted using R version 3.2.2 (R Core Team, 2013). To examine the effects of irrigation, maize variety, and organic soil amendments over three sampling dates, we fit general linear mixed effect models with block as the random effect, and ran analysis of variance, followed by pairwise comparisons using lsmeans. To ensure all data fit a normal distribution, transformation of log (x+1) were used for three response variables: NO<sub>3</sub><sup>-</sup>, microbial biomass C, and total soil C. Due to multiple zeros,

NH<sub>4</sub><sup>+</sup> data was analyzed for presence-absence using logistic regression. To analyze the activity of the seven enzymes, non-metric multidimensional scaling (NMDS) was used in the metaMDS package, using a Euclidean distance matrix, with random starting configurations. As three dimensions did not significantly further reduce stress, two dimensions are reported and goodness of fit was greater than 0.70 for all three ordinations. To test if differences in environmental soil data correlated to differences in EEA, an analysis of variance was conducted via a PerMANOVA using Adonis.

### **3. Results**

#### **3.1 Soil moisture and nutrients**

Averaged over the three sampling dates, manure significantly increased gravimetric water content over the control by 15% ( $p=0.037$ ) (Fig. 1). There was no significant interaction of soil amendment and irrigation level on soil moisture measurements. In July, at the height of the drought period, the limited irrigation treatment decreased soil gravimetric water content by 49% from the full irrigation ( $p=0.009$ , pairwise comparison). Soil amendments altered the percent of total soil C, the percent of total soil N, and the available P content, but not mineral N pools or soil pH (Table 3). Though manure had no impact on total soil C, biochar increased total soil C to 2.67%, an equivalent of an 80% increase over the control of 1.49% C ( $p<0.001$ ) (Table 3). Manure significantly increased total soil N by 9.7% over the control ( $p<0.001$ ) (Table 3). Over time, the percent total soil N accumulated in July ( $p<0.01$ ) and then decreased again in September ( $p<0.001$ ) (Table 3). Soil NH<sub>4</sub><sup>+</sup> and NO<sub>3</sub><sup>-</sup> levels were unaffected by soil amendments. Levels of NH<sub>4</sub><sup>+</sup> changed only over time, with undetectable NH<sub>4</sub><sup>+</sup> levels in June, and higher values in July and September ( $p<0.001$ ) (Table 3). Soil NO<sub>3</sub><sup>-</sup> levels differed between irrigation treatments and over time. The primary difference between irrigation treatments occurred in July, when soil NO<sub>3</sub><sup>-</sup> in the fully irrigated plots was significantly lower than in the other plots ( $p=0.018$ ) (Table 3). For soil available P, biochar had no effect, but manure increased available P by 45% over the control, averaged over time

( $p < 0.001$ ) (Table 3). Available P also varied over time between the irrigation treatments: from June to July available soil P increased by 39% in the fully irrigated plots ( $p = 0.055$ , pairwise comparison) and by 81% in the limited irrigation plots ( $p < 0.001$ , pairwise comparison) (Table 3).

### 3.2 Microbial biomass

Soil amendments significantly impacted MBC ( $p = 0.027$ ) with major differences between biochar and manure ( $p = 0.031$ , pairwise comparison) (Table 3). Manure significantly increased MBC by 15% over the control, while biochar decreased MBC slightly over the control (Table 3). Over time and irrigation treatments, between June and July MBC increased by 82% under full irrigation and by 33% under limited irrigation. In September, MBC decreased to similar levels under limited and full irrigation. MBN did not significantly differ by soil treatment ( $p = 0.093$ ), though manure maintained slightly higher MBN than the biochar treatment (Table 3). For MBN there was a significant interaction between irrigation and time, which occurred only in July. Averaged over soil amendment, in the fully irrigated plots MBN increased by 60.6% in July, while the limited irrigation decreased MBN by 46.2% in July (Table 3). In September, after the restart of irrigation, the MBN in the limited and fully irrigated plots increased by an average of 122% from July ( $p < 0.001$ ) (Table 3). These variable changes in MBC and MBN caused shifts in microbial C:N ratios with irrigation level, with the highest C:N ratios under limited irrigation in July ( $p < 0.001$ ) (Table 3). Soil amendments did impact microbial C:N ratios significantly, as averaged over other variables, microbial C tended to be higher in the manure plots ( $80.59 \mu\text{g g}^{-1}$  dry soil) than in the control ( $69.81 \mu\text{g g}^{-1}$  dry soil) or biochar plots ( $58.78 \mu\text{g g}^{-1}$  dry soil) (Table 3).

### 3.3 Potential extracellular enzymatic activities

Potential EEA (Table 2 for abbreviations) differed between soil amendments ( $p = 0.001$ ) and over time ( $p = 0.001$ , PerMANOVA). EEA varied in response to biochar treatment, but was not significantly impacted

by either manure nor limited irrigation treatments. Averaged over irrigation treatment and time, biochar increased AG, CB, and NAG activities by 186%, 70%, 571% over the controls, respectively (Fig. 2). Simultaneously, biochar decreased BG and PHOS overall activity by 41% and 43% from the controls, respectively (Fig. 2). There was no significant impact of biochar on LAP or XYL activity, but there was an impact of time and an amendment x time interaction. Averaged over amendment treatment, PHOS activity decreased by 50.2% and LAP increased by 20.3% in July (Fig. 2a and 2b). In September, XYL activity increased in biochar plots compared to the other amendment treatments (Fig. 2c). Further NMDS analyses revealed correlations between EEA and specific soil properties and microbial biomass (Fig. 3). Across all sampling dates in the biochar plots, the percent total soil C significantly correlated with increased AG, CB, and NAG activities, and lower slightly microbial biomass significantly correlated with the lower BG and PHOS activities (Fig. 3, PerMANOVA). Though amendments had no impact on LAP, increases in its activity correlated with high percent total soil N in July (Fig. 3b) and high  $\text{NH}_4^+$  in September (Fig. 3c).

### 3.4 Maize yield and biomass

Maize grain yield, harvest index, total dry biomass, and percent cob fill were not significantly impacted by soil amendment ( $p=0.881$ ) nor irrigation treatment ( $p=0.211$ ) averaged over other treatments (Fig.4). The limited irrigation showed slight trends toward decreasing yield by  $1.16 \text{ Mg ha}^{-1}$  ( $p=0.212$ ) (Fig. 4), slightly decreasing harvest index by 0.06 ( $p=0.331$ ), and slightly decreasing total dry biomass by 2.8% ( $0.29 \text{ Mg ha}^{-1}$ ) ( $p=0.299$ , pairwise comparisons). The lowest grain yield occurred in the biochar plots under limited irrigation ( $9.11 \text{ Mg ha}^{-1}$ ) and highest was the biochar plots under full irrigation ( $11.90 \text{ Mg ha}^{-1}$ ) (Fig. 4). The percent cob fill was significantly higher (10.75%) with the drought adapted maize hybrid (P8954AM) under limited irrigation ( $p=0.044$ ). This was the only significant difference observed between the two maize hybrids; other measurements are averaged across the hybrids.

## **4. Discussion**

### **4.1 Soil moisture and nutrients**

Manure increased gravimetric moisture content, while biochar had no significant impact on moisture content, and overall effects on nutrients also proved highly variable between amendments. Despite the larger pore volume of the biochar, we observed greater moisture increases with manure amendments. Additionally, analysis of plant available water revealed no effect of amendment, as the treatment differences were less than 2% volumetric soil moisture (data not shown). This may be attributable to water held tightly in biochar micropores, reducing moisture availability (Downie et al., 2009). Biochar additions consistently increase water holding capacity in sandy soils (Basso et al., 2013), where increases in microporosity have larger impacts (Abel et al., 2013), but not necessarily in clayey soils. Alternatively, the null impact on soil moisture could be due to the hydrophobicity of the biochar, which can increase at pyrolysis temperatures over 300°C (Zornoza et al., 2016). Though the manure did increase soil moisture, perhaps due to the sandy clay loam texture of our agricultural soil, the observed effect of amendment on soil moisture did not result in impact on yield. Researchers have previously attributed such results to the surface application of the amendment, which may not have impacted soil moisture in the deep maize rooting zone, as indicated with other crop species (Borchard et al., 2012). Our results warrant further research on the effects of biochar porosity and hydrophobicity on plant available water.

Biochar and manure had contrasting effects on soil nutrients. The high C content of the biochar (72.7%), applied at a 2.5% rate (w/w), added up to 19.6 Mg-C ha<sup>-1</sup> to the soil C stock. This upholds previous laboratory studies of biochar recalcitrance and C sequestration (Stewart et al., 2013) and corroborates one primary benefit of biochar additions in temperate systems (Jeffery et al., 2015; Spokas et al., 2012). Due to this high C addition, several previous temperate biochar field studies advise supplemental N

additions to minimize biochar induced N limitations (Brantley et al., 2015; Jones et al., 2012; Lehmann et al., 2003; Tammeorg et al., 2014; Zheng et al., 2012). However, at the 30 Mg ha<sup>-1</sup> addition rate, we observed no decrease in soil N pools, or increase in microbial N immobilization with the biochar plots. Though laboratory studies show biochar increased total soil N, when using a biochars with an initial N content of 0.61-1.22% (Ouyang et al., 2014; X. Wang et al., 2015), the negligible impacts on N dynamics in our field study matched the Jones et al. field trial (2012) with a pine biochar with an initial N content of 0.68%. These authors argue that the short term effects on soil nutrients observed in the laboratory were not observed in the longer-term, indicating that time since application may play a major role in biochar and N dynamics. As for other nutrients in our field trial, manure increased available P whereas biochar had no effect, despite containing a small amount of initial P (0.38%). In other field studies in temperate systems without P fertilization, biochar decreased extractable P (Nelson et al., 2011). This decline in available P has been attributed to soil alkalinization (Jay et al., 2015) and sorption onto biochar surfaces (Laird et al., 2010). As we measured no change in pH levels with soil amendments, the unchanging available P pool may have resulted from the small initial input balanced with soil microbial immobilization of P or rapid plant uptake (Anderson et al., 2011; Karer et al., 2013).

#### 4.2 Microbial biomass

Though previous studies using the chloroform-fumigation method measured significant sorption of extracted organic C and N onto pine-wood biochar (Jin, 2010), we did not measure any significant sorption due to biochar additions (data not shown); thus we present extraction values with no correction factor. As biochar did not enhance microbial biomass under limited irrigation, our results did not support our initial hypotheses. As observed with previous organic N field trials, manure increased MBC the most (Elzobair et al., 2016; Peacock et al., 2001; Witter et al., 1993). However, the observed slight decline in MBC with biochar addition contrasts with previous work in a maize agricultural field

with a slow-pyrolysis corn stover biochar (30 Mg ha<sup>-1</sup>) that demonstrated a doubling of microbial biomass (Domene et al., 2014). This 100% increase in microbial biomass with the corn stover biochar may have resulted from higher labile inputs from slow-pyrolysis production, but also includes a 1.77 correction factor used for biochar-sorbed dissolved organic C and N during the extraction, *versus* a 1.53 correction factor for the un-amended soils. In studies of other biochar types, observed increases in microbial biomass are often attributed to higher fungal and gram negative bacterial abundance (Gomez et al., 2014), related to changes in soil moisture and nutrient dynamics (Lehmann et al., 2011) that we did not observe in this study. Soil amendments did not impact microbial C:N ratios significantly, though microbial C:N tended to be higher in the biochar amended plots than in the other plots. Our findings of no impact on MBC:N ratios matches another field study with pine-wood biochar in a silt-loam soil cropped to corn (Brantley et al., 2015). Typically increases in microbial biomass are not observed as frequently with wood-derived biochars (Gul et al., 2015). Though we observed significant increases in MBC abundance with manure, and slight decreases with biochar, our analysis of soil enzymes revealed a more complex relationship between biochar and microbial function.

#### 4.3 Potential extracellular enzymatic activities

The observed increases in soil moisture, total C and N and microbial biomass did not necessarily improve soil productivity under limited irrigation as hypothesized. Similarly with EEAs, we observed no significant amendment x irrigation interaction. The only significant impact on EEA occurred in the biochar plots. The surprising lack of effects on EEA from the manure N and P addition actually conforms to previous results from a similar temperate maize system (Elzobair et al., 2016). In a study of microbial response to fertilizer treatments, Bolton et al., (1985) also demonstrated a more pronounced increase in EEA and microbial biomass with organic C, rather than N treatments. This also may begin to explain the more pronounced impact on EEA with the high C input from biochar additions.



Throughout the season, relative to the control, biochar amendments increased AG, CB, and NAG activity, decreased BG and PHOS activity, and had little to no impact on LAP and XYL activity. The increases in NAG, AG, and CB correlated with the high total soil C content in the biochar plots. Therefore, we hypothesize that these increases relate directly to higher organic C substrate in the soil, or to co-location and stabilization of C substrate and enzymes on the biochar surface. Since biochar caused a slight decrease in MBC, we may rule out increased EEA due to higher microbial biomass and thus higher production of enzymes. The extremely high increase in NAG (571%) compares to changes observed by Bailey et al. (2011) with a fast-pyrolysis switchgrass biochar amended to three soils, including an irrigated alkaline cropland soil. It is also possible that the high C content of the biochar induced higher relative microbial N requirements due to increased access to C substrate (Atkinson et al., 2010; M.L. Cayuela et al., 2013), thus increasing production of NAG, a C and N cycling enzyme. Previous research of AG and CB activities has primarily observed no impacts with pine biochar (Elzobair et al., 2016), and decreases with corn stover biochar (Jin, 2010; R. Wang et al., 2015). We hypothesize our results are related to feedstock type and pyrolysis, though other important factors that could account for these discrepancies are the charge of the specific enzymes tested and their interaction with the biochar surface (Elzobair et al., 2016).

Several possible mechanisms exist to explain the observed decreases in BG and PHOS activities in our field site with biochar addition. The potential sorption of enzymes or substrate on biochar surfaces may decrease EEA if the enzyme is denatured. If the enzyme adsorbs to a surface, causing a change in the morphology of the active site, then the enzyme may no longer function (Burns, 1982). Alternatively, as the decreases correlated with slightly lower microbial biomass C, the decline in EEA may simply indicate lower microbial biomass and production of enzymes. The observed decrease in BG aligned with several

previous studies: one in an alkaline irrigated crop soil amended with switchgrass biochar (Bailey et al., 2011), another in a maize field trial with corn stover biochar in a silt loam soil (Jin, 2010), and another laboratory study with sewage sludge biochar (Paz-Ferreiro et al., 2013). The decrease in PHOS, however, does not follow observed patterns with previous research in maize field trials with pine biochar (Brantley et al., 2015), or within a greenhouse maize biochar experiment (Masto et al., 2013), or in a majority of laboratory studies that demonstrate increases in PHOS with biochar additions, often associated with liming effects (Thies et al., 2015). Since our pine-wood biochar did not influence pH, as the calcareous soil already had a pH of 8.2, the observed decrease in PHOS did not relate to biochar liming the soil. However, since our biochar contained 0.38% P, this may have resulted in decreased production of PHOS initially if available P increased (Burns et al., 2013a). However, with no observed changes of available P in biochar plots, it is likely that this initial biochar-P was rapidly mineralized (Brantley et al., 2015). In another laboratory incubation with maize biochar in a fluvo aquic soil, Wang et al. (2015) explained that a decrease in PHOS activity may be due to sorption or blocking of the enzyme. This deactivation of enzymes may have also occurred in our biochar amended sandy clay loam soil. Further, we hypothesize that the pine biochar could also have interacted with signals for production of enzymes or for detection of substrate. This will require further research to elucidate direct mechanisms of decreased enzymatic activity.

The final two extracellular enzymes, LAP and XYL, showed no significant response to biochar additions, though other studies have found variable effects. In a 30 day lab incubation with an alkaline soils, Galvez et al. (2012) observed no impact of a 0.5% addition of green waste biochar on LAP. Similar to our study, the authors found the biochar amendment did not alter soil P, mineral N content, or microbial biomass. However, in a long term agricultural field trial, results differed from our study. Prommer et al (2012) observed a decrease in free amino acid production in similar a hard-wood biochar amended calcareous

soil, possibly due to adsorption or occlusion of LAP in biochar. This mechanism requires further research of biochar-enzyme sorption reactions. In our study LAP activity mirrored N dynamics, correlating to total soil N in July (Fig. 3b) and  $\text{NH}_4^+$  in September (Fig 3c). Despite this pattern and lack of impact from biochar, after a 90 day incubation researchers reported an increase in LAP activity with increasing biochar additions, suggesting that effect on N cycling enzymes depends upon biochar addition rate (R. Wang et al., 2015). Similarly, with measured XYL activities, results are variable. After a 36 day incubation with a loam soil, a 1.0% hardwood fast-pyrolysis biochar addition stabilized XYL against denaturation stress (Elzobair et al., 2015). Further, researchers observed a slight 16% increase in XYL activity with a 4% biochar addition in a silty arable soil with a pH of 6 (Bamminger et al., 2014). Since we observed no changes in LAP nor XYL, the variable results suggest that enzymatic response depends upon initial soil condition, including pH, biochar application rate, and feedstock type.

#### 4.4 Maize yield and biomass

In terms of crop response, limited irrigation caused a very slight reduction in grain yield and biomass (Fig. 4), yet there was no interaction with soil amendments. This result matches the maize field trial of Brantley, et al. (2014) with pine-wood chip biochar addition. In contrast, a maize field trial conducted by Rogovska et al. (2014) revealed an 11-55% increase in yield with a hardwood biochar after addition of high maize residue, likely due to biochar sorption of allelochemicals released by maize residues. Often in temperate agricultural systems, biochar does not impact crop yield in the first year, but on average has either a net positive or null effect (Biederman and Harpole, 2013). Yield has been shown to increase over time (Crane-Droesch et al., 2013), and thus we need longer-term results to reach a more definitive conclusion on the influence of biochar on maize yields.

#### 5. Conclusion

As predicted, pine-wood biochar significantly increased total soil C, confirming its sequestration potential. Although only manure increased gravimetric soil moisture, this did not result in enhanced maize yield under full or limited irrigation. However, since maize yield did not significantly decrease under 30% reduced irrigation, this experiment did support temporal limitation as an effective method to save water inputs in semi-arid agriculture. In terms of soil fertility, the manure added N and P to the soil, and increased microbial biomass, but had no effect on enzymatic activities. Contrastingly, biochar amendments slightly reduced microbial biomass C and had wide ranging impacts on EEAs. The biochar substantially increased NAG (571%) and decreased BG (-41%) activities, which corresponds to previous studies. However, the increases in AG (186%) and CB (70%), and decrease in PHOS (-43%) contrasted previous work. Elucidation of direct mechanisms related to biochar surface characteristics due to feedstock and pyrolysis conditions, its potential to stabilize or denature specific enzymes, and impact on optimal soil conditions for enzymatic activities, all require further research. This future work may help to specify how the organic amendment impacts soil microbial function, plant available nutrients, and potentially crop yield in temperate agroecosystems.

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## Captions

**Fig 1.** Gravimetric soil moisture response to biochar and manure amendments (applied to 15cm depth at 30 Mg ha<sup>-1</sup>) are compared to the control unamended soil. Panels represent the three sampling dates: (A) June 29 before irrigation treatments, (B) July 27 at the end of the drought period, and (C) September 01 before the final harvest. Limited irrigation treatment did not receive irrigation inputs for the month of July. Significant differences between full and limited irrigation occurred only in July. Data are the mean  $\pm$  1 SE are averaged over maize variety (n=8).

**Fig 2.** Response of seven extracellular enzymes (acronyms reported in Table 2) to biochar and manure amendments (applied to 15cm at 30 Mg ha<sup>-1</sup>) compared to the control sandy-loam agricultural soil. Data are the mean  $\pm$  1 SE, and are averaged over maize variety and irrigation that showed no significant differences (n=16), over three sampling dates on (A) June 29 before irrigation treatments, (B) July 27 at the end of the drought period, and (C) September 01 before the final harvest.

**Fig. 3.** Non-metric multidimensional scaling ordinations indicate how soil amendments relatively impact enzymatic activities and correlate to soil environmental variable vectors (black arrows) ( $p < 0.05$ ). Overall mean EEAs for seven enzymes are indicated with abbreviated labels (see Table 2). The points represent mean EEAs for each soil amendment treatment averaged over irrigation and corn variety (n=16) (manure as squares, biochar as triangles, and the control soil as open circles). Panel A, B and C correspond to the three sampling dates: (A) June before limited irrigation treatment began, (B) July, at the end of the limited irrigation, and (C) September before final maize harvest. Significantly correlated soil properties included mbc (microbial biomass carbon), mbn (microbial biomass n), soil N (total % soil N), soil C (total % soil C) and nh4 (ammonium) (units in Table 3). Plots are rotated so that the total soil C vector runs along the primary axis. Goodness of fit was  $>0.70$  for all ordinations.

**Fig 4.** Response of maize harvest grain yield (left panel) and dried whole plant biomass (right panel) to organic soil amendments applied at 30 Mg ha<sup>-1</sup>, data are presented as the mean  $\pm$  1 SE (n=8). No significant differences were found between the soil amendments or irrigation treatment at the  $p < 0.05$  level.

**Table 1**

Characteristics of the agricultural control soil and the two amendments, applied at a rate of 30 Mg ha<sup>-1</sup> and tilled to a depth of 15 cm in November of 2013. Surface area analysis was conducted using the Brunauer-Emmet-Teller method, using N<sub>2</sub> desorption. Values are mean  $\pm$  1 SE. No SE available for BET analysis (n=1) or for biochar bulk density (analyzed by Control Laboratories, Watsonville, CA).

Treatment	Total C (%)	Total N (%)	pH	$\rho_b$ (g cm <sup>-3</sup> )	CEC (nmol <sub>c</sub> kg <sup>-1</sup> )	Surface Area (m <sup>2</sup> g <sup>-1</sup> )	Pore surface area (m <sup>2</sup> g <sup>-1</sup> )	Pore volume (cm <sup>3</sup> g <sup>-1</sup> )
Biochar (B)	72.7 $\pm$ 2.30	0.5 $\pm$ 0.01	9.2	0.35	16.51 $\pm$ 0.53	232.72	60.40	0.16
Manure (M)	24.8 $\pm$ 0.64	1.5 $\pm$ 0.06	9.8	nd	46.91 $\pm$ 1.07	2.69	2.06	0.01
Control Soil (C)	1.49 $\pm$ 0.04	0.14 $\pm$ 0.002	8.7	1.35 $\pm$ 0.03	21.59 $\pm$ 1.21	27.50	18.76	0.03

Abbreviations: nd=no data



**Table 2**

The seven extracellular enzymes measured fluorometrically in an agricultural soil amended with biochar and manure, and their abbreviations, general functions and substrates (adapted from Alster et al. 2013).

Enzyme	Abbreviation	Function	Substrate
$\alpha$ -1,4- glucosidase	AG	Complex carbohydrates, starch and glycogen	4-MUB- $\alpha$ -D-glucopyranoside
$\beta$ -1,4-glucosidase	BG	Hydrolysis of cellulose (glucose)	4-MUB- $\beta$ -D-glucopyranoside
$\beta$ -D-cellobiohydrolase	CB	Hydrolysis of cellulose	4-MUB- $\beta$ -D-cellobioside
L-leucine aminopeptidase	LAP	Hydrolysis of leucine residues at end of peptides/proteins	L-Leucine-7-amido-4-methylcoumarin hydrochloride
$\beta$ -1,4-N-acetylglucosaminidase	NAG	Chitin degradation	4-MUB-N-acetyl- $\beta$ -D-glucosaminide
Phosphatase	PHOS	Reduction of organic P to phosphate groups	4-MUB-phosphate
$\beta$ -Xylosidase	XYL	Reduction of cellulose from xylan	4-MUB- $\beta$ -D-xylopyranoside
Fluorogenic moiety:			
4-Methylumbelliferone	MUB	Fluoresces with enzyme catalyzed substrate degradation	
7-Amino-4-methylcoumarin	MUC	Fluoresces with enzyme catalyzed substrate degradation	

Abbreviations: irrig = irrigation treatment, amend = soil amendment, time=sampling date (June, July, Sept), variety = variety of maize hybrid, nd = no data, ns = not significant, df=degrees of freedom.

**Table 3**

Comparison of soil biogeochemical properties, between full and limited irrigation with biochar, manure or the no amendment control, for each sampling date over the growing season. Means  $\pm$  1 SE are averaged over the two maize varieties, which showed no significant differences, (n=8). P-values reported at  $p < 0.10$  with significant values bolded when  $p < 0.05$  from a mixed effects ANOVA of irrigation, maize variety, and amendment over time.

Experimental Treatments:		Irrigation	Amendment	Sampling Month:	K Cl Extractable Mineral N				Fumigation Extractable Microbial Biomass				Elemental Content				Basic Properties						
					NO <sub>3</sub> <sup>-</sup> (ug g <sup>-1</sup> ) mean SE		NH <sub>4</sub> <sup>+</sup> (ug g <sup>-1</sup> ) mean SE		C (ug g <sup>-1</sup> ) mean SE		N (ug g <sup>-1</sup> ) mean SE		C:N Ratio mean SE		Total C (%) mean SE		Total N (%) mean SE		Available P (ppm) mean SE		Water Content mean SE		pH mean SE
Full	Biochar	June	48.38	9.99	0.00	0.00	38.17	4.50	5.35	0.87	9.12	2.14	2.86	0.30	0.15	0.00	12.77	2.07	14.77	0.63	8.55	0.09	
		July	11.46	2.43	0.00	0.00	88.09	13.79	11.52	1.82	7.90	0.63	2.46	0.11	0.14	0.00	16.99	1.79	15.04	0.67	nd	nd	
		Sept	4.45	1.00	2.80	0.61	60.13	6.50	14.88	0.81	3.96	0.25	2.62	0.14	0.13	0.00	nd	nd	11.51	0.51	8.69	0.10	
	Control	June	48.61	7.77	0.00	0.00	58.34	4.97	10.77	2.20	6.48	0.96	1.50	0.16	0.14	0.00	9.37	2.09	13.71	0.62	8.66	0.04	
		July	21.55	6.81	0.00	0.00	94.87	11.83	11.60	2.60	8.30	0.82	1.40	0.09	0.15	0.00	16.46	3.08	12.86	0.47	nd	nd	
		Sept	5.12	0.86	2.65	0.61	73.16	13.09	17.60	2.66	4.05	0.23	1.40	0.16	0.13	0.00	nd	nd	10.40	0.60	8.68	0.07	
	Manure	June	43.59	8.25	0.00	0.00	64.93	6.70	8.79	1.20	8.04	1.28	1.38	0.05	0.15	0.00	21.44	2.37	15.35	1.30	8.60	0.07	
		July	10.60	2.25	0.00	0.00	110.87	15.77	16.88	2.02	6.32	0.63	1.59	0.12	0.15	0.00	26.71	2.16	14.68	0.31	nd	nd	
		Sept	3.82	0.62	2.83	0.62	72.35	7.42	16.59	1.13	4.30	0.22	1.39	0.07	0.14	0.00	nd	nd	11.66	0.56	8.75	0.06	
Limited	Biochar	June	46.89	9.67	0.00	0.00	44.36	3.69	7.47	0.79	6.29	0.64	2.52	0.14	0.14	0.01	15.38	3.27	15.48	1.78	8.51	0.05	
		July	45.34	11.50	0.22	0.10	67.56	8.55	4.26	2.08	14.92	3.72	2.97	0.17	0.15	0.00	22.33	1.94	7.55	0.34	nd	nd	
		Sept	14.35	3.05	2.84	0.62	54.37	4.63	13.47	0.61	4.03	0.28	2.61	0.17	0.13	0.01	nd	nd	10.96	0.33	8.61	0.06	
	Control	June	39.96	8.46	0.00	0.00	59.82	7.02	8.60	0.69	6.93	0.62	1.46	0.05	0.13	0.01	14.57	1.45	14.42	2.03	8.64	0.06	
		July	37.40	12.17	0.32	0.10	71.82	4.51	4.60	0.93	16.03	3.10	1.62	0.09	0.14	0.00	26.43	3.92	6.65	0.49	nd	nd	
		Sept	17.83	6.42	2.85	0.63	60.88	7.19	12.74	1.68	5.26	0.94	1.53	0.07	0.13	0.00	nd	nd	9.45	0.37	8.65	0.07	
	Manure	June	41.04	5.90	0.00	0.00	66.43	8.58	9.34	1.72	7.74	0.80	2.04	0.25	0.16	0.01	14.73	2.15	17.95	2.56	8.70	0.06	
		July	44.63	9.44	0.14	0.07	89.33	9.68	4.82	1.39	15.47	1.85	1.67	0.06	0.16	0.00	33.96	2.02	7.65	0.22	nd	nd	
		Sept	10.35	2.52	2.95	0.54	79.61	4.70	17.33	0.66	4.59	0.18	1.55	0.10	0.14	0.01	nd	nd	10.55	0.38	8.67	0.07	
		df		P-values																			
		irrig	1	0.043		ns		ns		0.039	0.011		ns		ns		ns		ns		ns		ns
amend	2	ns		ns		0.027		0.093	ns		<0.001		<0.001		<0.001		0.037		ns		ns		
variety	1	ns		ns		ns		ns	ns		ns		ns		ns		ns		ns		ns		
time	2	<0.001		<0.001		<0.001		<0.001	<0.001		ns		<0.001		<0.001		<0.001		0.077		ns		
irrig x amend	2	ns		ns		ns		ns	ns		ns		ns		0.086		ns		ns		ns		
irrig x variety	1	ns		ns		ns		ns	ns		ns		ns		ns		ns		ns		ns		
amend x variety	2	ns		ns		ns		ns	ns		ns		ns		ns		ns		ns		ns		

irrig x time	2	<b>&lt;0.001</b>	ns	ns	<b>&lt;0.001</b>	<b>&lt;0.001</b>	ns	ns	<b>0.013</b>	<b>&lt;0.001</b>	ns
amend x time	4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
variety x time	2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
irrig x amend x variety	2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
irrig x amend x time	4	ns	ns	ns	ns	ns	<b>0.003</b>	<b>0.037</b>	ns	ns	ns
irrig x variety x time	2	0.054	ns	ns	ns	ns	ns	ns	ns	ns	ns
amend x variety x time	4	ns	ns	ns	ns	ns	ns	0.052	ns	ns	ns
irrig x amend x variety x time	4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Abbreviations: irrig = irrigation treatment, amend = soil amendment, time=sampling date (June, July, Sept), variety = variety of maize hybrid, nd = no data, ns = not significant, df=degrees of freedom.







